



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/68, 1/70	A1	(11) International Publication Number: WO 94/03635 (43) International Publication Date: 17 February 1994 (17.02.94)
(21) International Application Number: PCT/GR92/00015 (22) International Filing Date: 4 August 1992 (04.08.92) (71) Applicant: INSTITUTE OF MOLECULAR BIOLOGY & BIOTECHNOLOGY [GR/GR]; P.O. Box 1527, GR-711 10 Heraklion (GR). (72) Inventors: TAVERNARAKIS, Nectarios ; Matzapetaki 21, GR-Agios Ioannis (GR). HATZIDAKIS, George ; Geronymaki 32, GR-713 06 Heraklion (GR). KRAMBOVITIS, Elias ; Papanastasiou 175, GR-Heraklion (GR). (74) Agent: PAPACONSTANTINO, Helen, G.; 2 Coumbari Street, GR-106 74 Athens (GR).		(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i>
(54) Title: RAPID AMPLIFICATION AND DETECTION OF NUCLEIC ACIDS (57) Abstract Disclosed herein are methods, primers, probes, and kits for the rapid amplification and detection of nucleic acids. In a preferred embodiment, a target nucleic acid sequence in a sample is amplified by: 1) adding nucleoside triphosphates, primer pairs comprising two oligonucleotide primers, and a nucleic acid polymerase to the sample; 2) denaturing the target nucleic acid sequence to form separate strands; and 3) maintaining a reaction temperature in a range from 68 °C to 80 °C and appropriate reaction conditions wherein the following cycle occurs: the primers hybridize to the target strands, primer extension products are formed, the extension products separate to become templates for the primers, and new extension products are formed. In an alternative embodiment, the amplification occurs at two different temperature ranges. Primer extension products are formed in a temperature range from 68 °C to 82 °C, and the products are separated by raising the temperature to range of 88 °C to 96 °C. Detection of the amplified sequences occurs by using some biotin-labeled nucleoside triphosphates, which produces nucleotide sequences that are copies of the target sequence and that contain one or more biotin-labeled nucleotides. The amplified sequences are detected by contacting the sample with immobilized probes and then detecting the presence of the biotin. A two-stage amplification process is also provided wherein: 1) the probe-target sequence complexes are contacted with a first moiety that binds to biotin; 2) a second moiety, comprising biotin bound to a detectable moiety is added, wherein the biotin in the second moiety binds to the first moiety; and 3) detecting or measuring the detectable moiety. In a particularly preferred embodiment, primer pairs and a probe are disclosed for use when the target nucleic acid is HIV-1 DNA.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LJ	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

- 1 -

RAPID AMPLIFICATION AND DETECTION OF NUCLEIC ACIDSFIELD OF THE INVENTION

This invention relates to the rapid amplification and detection of nucleic acids. In particular, the invention provides improved methods for amplifying small amounts of nucleic acids in a sample in which the
5 amplification steps are conducted at the same temperature or, alternatively, at only two different temperatures. In addition, the invention provides improved methods for detecting the amplified nucleic acids in which the detection signal is boosted. Related probes and test kits
10 are also provided. The invention is expected to be useful in a wide variety of fields, including scientific, clinical, and forensic analysis. In one specific embodiment, it is particularly useful in the detection of human immunodeficiency virus type 1 (HIV-1), the causative
15 agent of AIDS.

BACKGROUND OF THE INVENTION

The ability to detect exceedingly small amounts of a nucleic acid in a sample generally requires the amplification of the amount of the target nucleic acid.
20 This is especially important for the detection of human retroviruses, where positive samples may contain only 5-10 target molecules in 10^6 cells.

The preferred method for amplifying target DNA has been the polymerase chain reaction (PCR) technique. The
25 technique has been described in U.S. Patent Nos. 4,683,195, issued July 28, 1987 to Mullis, et al., 4,683,202, issued

- 2 -

July 28, 1987 to Mullis, 4,800,159, issued January 24, 1989 to Mullis, et al., 4,889,818, issued December 26, 1989 to Gelfand, et al., and 4,902,624, issued February 20, 1990 to Columbus, et al., all of which are incorporated herein by
5 reference.

In general, the PCR reaction involves the use of a pair of specific oligonucleotide primers to initiate DNA synthesis on a target DNA template. Two oligonucleotide primers are used for each double-stranded sequence to be
10 amplified. The target sequence is denatured into its complementary strands. Each of the primers, which are sufficiently complementary to a portion of each strand of the target sequence to hybridize with it, anneals to one of the strands. The primers are extended, using nucleosides
15 in the sample and a polymerization agent, such as heat-stable Taq DNA polymerase. This results in the formation of complementary primer extension products, which are hybridized to the complementary strands of the target sequence. The primer extension products are then separated
20 from the template strands, and the process is repeated until the desired level of amplification is obtained. In subsequent cycles, the primer extension products serve as new templates for synthesizing the desired nucleic acid sequence.

25 By repeating the cycles of denaturation, annealing, and extension, the original target DNA can be amplified exponentially according to the formula 2^n , where n is the number of cycles. In theory, 25 cycles, for example, would result in a 3.4×10^7 -fold amplification. However, since
30 the efficiency of each cycle is less than 100%, the actual amplification after 25 cycles is about $1-3 \times 10^6$ -fold. The

- 3 -

size of the amplified region is generally about 100-400 base pairs, although stretches of up to 2 kb can be amplified. See Keller and Manak, DNA Probes (New York: Stockton Press, 1989), pgs. 215-216.

5 The three basic steps of the PCR reaction -- denaturation, annealing, and extension -- are driven and controlled by the temperature of the reaction mixture, with each step occurring at a different temperature. Somewhat different temperature ranges are disclosed for each of the
10 three steps in the above-referenced patents. However, as time passed, those skilled in the art have settled on fairly standard temperatures for each of the steps in the cycle. Thus, Gelfand, et al., discloses a denaturing temperature range of about 90°-105°C, preferably 90°-100°C,
15 an annealing temperature range of about 35°-65°C, preferably 37°-60°C, and an extension temperature range of about 40°-80°C, preferably 50°-75°C. For Taq polymerase, which is the overwhelmingly preferred polymerase for the PCR reaction, Gelfand, et al., refers to an annealing
20 temperature range of about 45°-58°C and an extension temperature range of about 65°-75°C. Columbus, et al., which is directed to a temperature cycling cuvette for use in PCR, refers to temperature ranges of 92°-95°C for the denaturing step, 50°-60°C for the annealing step, 70°C for
25 the extension step. Keller and Manak, cited above, refer to a denaturation temperature of about 93°C, an annealing temperature of 37°-55°C, and a primer extension temperature of 70°C.

 The PCR technique has been modified to permit the
30 amplification of viral RNA. See Murakawa, et al., DNA, 7:287-295 (1988), which is incorporated herein by

- 4 -

reference. The article discloses the amplification of sequences from HIV-1 RNA templates for the identification of HIV-1 in peripheral blood and tissue samples obtained from AIDS and ARC patients. Total nucleic acid is isolated from infected cells, and the DNA is digested with RNase-free DNase so that it does not contribute to the final PCR product. A cDNA copy of a target sequence of the viral RNA is synthesized, using the PCR primers and reverse transcriptase. The one primer complementary to the RNA serves to initiate cDNA synthesis.

Several different formats have been used for the detection of PCR products. Generally, a radioactive or nonradioactive labeled probe that is complementary to the target sequence is used. The hybridization of the probes to the amplified target sequence, and the subsequent detection of the labeled moiety results in the detection of the target sequence. Nonradioactive labeled probes are generally more desirable because they obviate the need for special handling procedures. However, they may not generate as intense a signal, or the signal may be obscured by background "noise." Thus, there is a need for enhancing the intensity of the signal in such probes.

The PCR technique is a revolutionary one, and it is widely used. Nevertheless, it does have significant drawbacks. The most serious of these is nonspecific hybridization, which results in false positives. Avoiding nonspecific hybridization requires ultrapure reagents. Unfortunately, for most clinical and diagnostic applications, it is desirable to use "dirty" samples, which presents a major problem, unless time-consuming and expensive sample preparation is undertaken.

- 5 -

Another important drawback to the PCR technique is the time involved in amplification. Although the usual six hour time period for PCR is far superior to an alternative technique such as cloning, which can take days or weeks, it
5 would still be desirable to cut amplification time by one-half to two-thirds.

The present invention overcomes these drawbacks of the PCR technique, and it provides an improved detection system. The invention provides methods for the rapid
10 amplification and detection of nucleic acids in which the denaturing, annealing, and extension steps occur all at the same temperature or, alternatively, at only two different temperatures, thus providing for faster cycling. In addition, the invention provides amplification methods
15 where the annealing temperature is higher than the prior art temperatures, thus eliminating nonspecific hybridization. Finally, the invention provides improved methods of detecting the amplified nucleic acids through a two-stage signal amplification.

20

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for amplifying a target nucleic acid sequence in a sample.

Another object is to provide methods for detecting
25 or measuring the presence of a target nucleic acid sequence in a sample.

- 6 -

Still another object of the invention is to provide a kit for detecting or measuring the presence of a target nucleic acid sequence in a sample.

A further object of the invention is to provide
5 primer pairs for use in amplifying a nucleic acid sequence of HIV-1 DNA.

A still further object is to provide a probe for use in detecting a nucleotide sequence complementary to a target nucleic acid sequence of HIV-1 DNA.

10 Additional objects and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by the practice of the invention. The objects and advantages of the invention will be attained by means of
15 the instrumentalities and combinations particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides a method for
20 amplifying a target nucleic acid sequence in a sample. The sequence is part of a nucleic acid, which the sample is suspected of containing. Preferably, the target nucleic acid is DNA, most preferably HIV-1 DNA.

Nucleoside triphosphates, primer pairs consisting
25 of two oligonucleotide primers, and a nucleic acid polymerase are added to the sample. Each primer is an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target

- 7 -

sequence. It is effective as an initiator for nucleoside polymerization. If double stranded, the nucleic acid in the sample is denatured so that separate strands of the target nucleic acid sequence are formed. Preferably, the
5 denaturing is accomplished by heating the sample to about 95°C.

A reaction temperature in a range from about 68°C to about 80°C (preferably about 75°C) and appropriate reaction conditions are maintained so that the following
10 cycle occurs. First, the primers anneal (hybridize) to the separate strands of the target sequence. The primers and the nucleic acid polymerase initiate the synthesis of primer extension products, formed by the nucleosides attaching to the primer and forming a polymer, using the target sequence
15 strand as a template. Thus, the primer extension products are annealed or hybridized to the strands. The primer extension products are then separated from the strands to become templates for the primers, and the cycle is repeated, with new primer extension products being formed.
20 The reaction is allowed to continue for a sufficient number of cycles until the desired amplification of the target nucleic acid sequence has been accomplished. The primers are chosen so that they bind strongly to the strands of the target sequence, but the extension from each primer binds
25 weakly to the strand.

In an alternative embodiment of the invention, the cycling occurs at two different temperatures. The reaction temperature is maintained in a range from about 68°C to about 82°C (preferably about 70°C) and appropriate reaction
30 conditions are maintained to form the primer extension products. These are separated from the strands by raising

- 8 -

the temperature to a range of about 88°C to about 96°C (preferably about 90°C) to produce single-stranded molecules. The temperature is then lowered back to the previously mentioned range, permitting the primers to
5 anneal to the single-stranded molecules and primer extension products to be synthesized, using the single-stranded molecules as templates. The temperature cycling is repeated a sufficient number of times to obtain the desired amplification of the target nucleic acid sequence.

10 The invention also provides methods for detecting or measuring a target nucleic acid sequence in a sample, based upon the amplification methods discussed above. In these methods, some of the nucleoside triphosphates are biotin-labeled. This produces nucleotide sequences that
15 are copies of the target sequence which contain one or more biotin-labeled nucleotides. Such sequences are rendered single-stranded and contacted with immobilized probes. Each of the probes is a single-stranded polynucleotide attached to a solid support and capable of hybridizing with
20 one of the single-stranded nucleotide sequences. The probes are contacted with the sample for a sufficient time and under appropriate hybridizing conditions to permit the polynucleotides to hybridize with the single-stranded nucleotide sequences. This forms bound complexes comprised
25 of both of these entities. The presence of the biotin in the bound complexes is then detected or measured. Preferably, the biotin is detected by contacting the bound complexes with a detectable moiety that binds to biotin, such as avidin-horseradish peroxidase, and then detecting
30 the detectable moiety, i.e., the horseradish peroxidase.

- 9 -

In an alternative and preferred embodiment, the detecting step is accomplished by contacting the bound complexes with a first moiety that binds to biotin, such as avidin. A second moiety, comprising biotin bound to
5 detectable moiety, such as horseradish peroxidase, is then added. The biotin in this second moiety binds to the first moiety. The detectable moiety is then detected. This provides a two-stage amplification of the signal.

The invention further provides kits for detecting
10 or measuring the presence of a target nucleic acid sequence in a sample. The kits contain biotin-labeled nucleoside triphosphates and a primer pair specific for the particular target sequence sought to be detected or measured. Preferably, the kit further comprises a probe that is
15 specific for the target sequence. It may also contain a nucleic acid polymerase, reagents for detecting or measuring the biotin, denaturing reagents, and/or controls.

In a preferred embodiment of the kit, the target nucleic acid sequence is HIV-1 DNA. The primer pair
20 comprises the nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5' (SEQ ID NO: 2)

and the probe comprises the nucleotide sequence

5' TTAAACACC ATGCTAAACA CAGT 3'. (SEQ ID NO: 3)

- 10 -

DETAILED DESCRIPTION OF THE INVENTION

Reference will now made in detailed to the presently preferred embodiments of the invention which, together with the following examples, serve to explain the principles of the invention.

The invention provides improved methods for amplifying and detecting nucleic acids. The target nucleic acid may be single or double-stranded DNA or RNA from any organism. Such organisms include plants, animals, and microorganisms, such viruses, viroids, mycoplasma, bacteria, and fungi. The viruses include DNA and RNA viruses. In a preferred embodiment, the viruses are retroviruses, and in a particularly preferred embodiment, they are the AIDS-related viruses (including HIV-1 and HIV-2). The term "animals" includes mammals, and, in a preferred embodiment, the target nucleic acid is mammalian nucleic acid, such as mitochondrial or genomic DNA or the various types of RNA. Such nucleic acid further includes human cellular oncogene sequences and human structural gene sequences.

The sample may be anything that contains nucleic acid, obtained from a source by techniques known to those skilled in the art. It may be further processed by known techniques, such as being subjected to extraction procedures, to render it in a form usable in the method of the invention. For example, the method of the present invention can be applied to determine if a patient has been infected by a virus. A sample of body fluid or tissue that contains the virus is obtained from the patient. In this case, the sample should contain cells that are capable of

- 11 -

being infected by the virus or should contain body fluid in which the virus or viral nucleic acid is known to accumulate. The viral nucleic acid is extracted from the sample by known techniques.

5 A target nucleotide sequence within the target nucleic acid is selected so as to provide a target of sufficient specificity and uniqueness in order to be identified and distinguished from other nucleic acid molecules in the sample. Among other things, it will be
10 selected on the basis of a nucleotide base sequence specific to the organism. For many viruses, in particular those that mutate at high rates, it is advisable to select a target sequence from a well-conserved region of the viral genome.

15 The target nucleotide sequence may consist of from about 50 to about 4000 base pairs, but preferably consists of about 55 to about 200 base pairs, and most preferably consists of about 60 to about 100 base pairs. In the case of HIV-1 DNA, the preferred target sequences are the
20 nucleotide sequence 1317-1379 of PHXB2 and 331-530 of PHXB2, depending upon which primer pair of the invention is used to amplify the sequence.

 In the preferred embodiment of the invention, the target sequence is amplified in a reaction that occurs at a
25 single temperature, after the initial denaturation of the nucleic acid, in contrast to the three different temperatures in the PCR technique. We call this method the Continuous Enzyme Reaction (CER). In the first step, certain reagents are added to the sample that contains the
30 target nucleic acid. In particular, nucleoside

- 12 -

triphosphates, including biotinylated nucleotide triphosphates, primer pairs, and a nucleic acid polymerase are added. Preferably, they are added as part of a buffered solution. Each primer is an oligonucleotide
5 having a region that is complementary to and hybridizes with a different strand of the target sequence. It is effective as an initiator for nucleoside polymerization. Preferably, the length of each primer is approximately 15-25 nucleotides.

10 The target nucleic sequence, if it is double-stranded, is then separated into single strands by known techniques. Preferably, the nucleic acid is denatured by heating the sample to about 95°C. The target sequence may be denatured before the other reagents are added to the
15 sample, although it is preferable to denature it afterwards.

The sample is then maintained under appropriate reaction conditions to permit the following cycle to occur:
(1) each of the oligonucleotide primers anneals
20 (hybridizes) to each of the strands of the denatured target sequence; (2) primer extension products are synthesized from the nucleoside triphosphates, with the synthesis being initiated by the primer and catalyzed by the nucleic acid polymerase; and (3) a portion of the extension products,
25 which are hybridized to the strands of the target sequence, separate spontaneously from the strands. The separated extension products become templates for the primers, and the cycle is repeated. Such reaction conditions include maintaining the reaction temperature somewhere in the range
30 from about 68°C to about 80°C. Preferably, the temperature is maintained at about 75°C.

- 13 -

The reaction is permitted to proceed for a sufficient period of time so that the cycle is repeated about 10 to about 25 times. This will occur over a time ranging from about 1.0 hours to about 2.5 hours.

5 Generally, the longer reaction time is preferred.

The nucleoside triphosphates are readily available commercially or can be synthesized by those skilled in the art. When the method of the invention is applied to the amplification of DNA, deoxyribonucleoside triphosphates are
10 used.

Most preferably, some of the nucleoside triphosphates or deoxyribonucleoside triphosphates are labeled with biotin. This permits the incorporation of a reporter molecule into the amplified sequences, which can
15 be detected by the means described below. Such biotin-labeled nucleoside triphosphates can be synthesized by known techniques or are commercially available. The preferred biotin-labeled nucleoside triphosphate is Bio-dUTP. The labeled nucleosides are used in a concentration
20 of about 10 μ M to about 5mM, and preferably from about 0.1 mM to about 0.5 mM.

The polymerizing agent is any polymerase for nucleic acid. Preferably, it is Tag polymerase, which is heat stable under these reaction conditions.

25 The primer pairs consist of two oligonucleotide primers. Each primer is an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target sequence, and is effective as an

- 14 -

initiator (primer) for nucleoside polymerization. The primers of the invention are selected so as to meet two requirements. First, they must bind strongly to the strands of the target sequence to allow binding at a
5 temperature higher than the temperature preferred in the literature. Second, the product from the extension of the primers, which is synthesized from the nucleosides in the presence of the polymerase, is the least stable of a series of products which were initially selected and more likely
10 to dissociate spontaneously to single-stranded form, thus itself becoming a target sequence of the appropriate primer. As used herein, the term "bind strongly" and variations thereof means adequately stable single-stranded
16DNA-primer hybrids are formed at the high temperature of
15 the invention.

This is accomplished by choosing primers where the ratio of G and C to A and T in the primers ranges from about 1.5:1 to about 3.0:1. Preferably, the ratio is about 3.0:1. This is confirmed by the temperature of
20 dissociation (T_d) of the primer pair and the melting temperature (T_m) of the extension products. The T_d of the primer pair is calculated from the formula $T_d = 4 \cdot (C+G) + 2 \cdot (A+T)$. The T_m , which is the temperature at which the extension products are denatured, is calculated from the
35 formula:

$$T_m = 81.5^\circ + 16.6 \log C_i + 0.41 (\% C+G) - 0.72 (\% \text{ formamide}) - 82^\circ/n - 1.5 (\% \text{ mismatch})$$

where C_i is the ionic strength of the reaction solution and n is the number of nucleotides.

- 15 -

For HIV-1 DNA, the following specific primers, which we call HOPE 1 and HOPE 2, are preferred:

HOPE 1: 5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

HOPE 2: 3' CCCCCCTGTA GTTCGTCGG 5' (SEQ ID NO: 2)

5 These primers produce HIV-1 DNA sequences in positions 1317-1379 of the plasmid PHXB2, which are 63 base pairs long. The Td for HOPE 1 is 62°C and that for HOPE 2 is 64°C. The Tm of the product, i.e., the primer extension, is 76.7°C.

20 The use of primers meeting the above-stated conditions permits the maintenance of temperature and other reaction conditions whereby the amplification cycle occurs at a single temperature rather than three different temperatures. In order for the reaction to occur
25 efficiently in terms of yielding double-stranded target DNA, the following reaction conditions should be considered:

1) The temperature should be high enough so that a substantial portion of the target DNA is found in single-
30 stranded form, available for primer hybridization (the activity of the polymerase is a limiting factor).

2) The concentration of the primers should be high enough so as to favor single-stranded DNA-primer hybrid formation.

- 16 -

3) The GC content of the primers should be such as to allow an adequately stable single-stranded DNA-primer hybrid to be formed at the high temperature chosen.

4) The primer extension product, on the contrary,
5 should be as unstable as possible so that the probability for the two strands to dissociate spontaneously is as high as possible.

5) The polymerizing agent should be able to efficiently polymerize dNTPs at the high temperature chosen
10 and also to incorporate the dNTP analogue used as a label onto DNA strands.

Generally, the cycle of primer annealing, primer extension, and extension product denaturing occurs from about 10 to about 25 times. The time period for this to
15 occur is approximately 0.5 hours to overnight.

In an alternative and preferred embodiment, asymmetric concentrations of the primers are used. That is, the concentration of one of the primers exceeds that of the other by a factor of about 10^3 to 1, and preferably by
20 about 10^2 to about 1. This permits the build up of only one instead of two primer extension products. Therefore, there is only a limited risk of DNA-DNA complexing during hybridization. Instead, the product DNA will bind to the primer or the probe that is used for detection of the
25 amplified DNA. This enhances both the specificity and sensitivity of the assay.

- 17 -

When the nucleic acid to be amplified is RNA, certain modifications are made. The RNA template in infected cells is either viral mRNA or packaged virion RNA. Total nucleic acid is isolated in crude form by known
5 methods, or it can be purified by, for example, phenol-chloroform extraction. Residual DNA may be digested with RNase-free DNase so that it does not contribute to the amplified sequences. A cDNA copy of a target
ribonucleotide sequence in the target RNA is synthesized,
10 using the primers and reverse transcriptase. The primer complementary to the target sequence serves to initiate cDNA synthesis. The single-stranded cDNA product can be amplified after heat inactivation of the reverse transcriptase and adjustment of the reaction conditions to
15 those of the method of the invention.

In an alternative embodiment of the invention, the amplification of the target nucleic acid sequence occurs at two different temperatures. The primer annealing and extension steps occur at one temperature, and the
20 denaturing of the primer extension products occurs at another temperature. We call this method the Accelerated Chain Enzyme Reaction (ACER).

In particular, the reaction temperature is maintained at a point in the range from about 68°C to about
25 82°C for primer annealing and extension. Preferably, the reaction temperature for these steps of the cycle is about 70°C. The primer extension products are separated from the target sequence strands or other extension products by raising the temperature to a range of about 88°C to about
30 96°C. This denatures the extension products from the template, producing single-stranded molecules that serve as

- 18 -

further templates when the temperature is lowered.
Preferably, the denaturing step occurs at a temperature of about 90°C.

The cycling between these two temperatures is
5 repeated about 10 to about 25 times. The reaction time for these steps is from about 0.5 hours to about 3 hours. Generally, the time is about 1.5 hours.

The preparation of the sample and the initial
reaction mixture is essentially the same as in the single
10 temperature method. The target nucleic acid sequence is also denatured in the same manner. This method also lends itself to the use of asymmetric concentrations of primers and to the amplification of RNA as discussed previously.

The specific primers for any given target sequence
15 are determined as discussed above with respect to the single temperature embodiment. The primers will have the same general characteristics mentioned above. For HIV-1 DNA, the previously mentioned HOPE 1 and HOPE 2 primers are preferred. In addition, two other primers, which we
20 designate NIDA 1 and NIDA 2, are preferred. These primers comprise the following sequences:

NIDA 1: 5' GACATCGAGC TTGCTAGAAG 3' (SEQ ID NO: 4)

NIDA 2: 3' GGTGACGAAT TCGGAGTTAT 5' (SEQ ID NO: 5)

They produce HIV-1 DNA sequences in positions 331-530
25 PHXB₂.

- 19 -

In another embodiment, the invention comprises a method for detecting or measuring a target nucleic acid in a sample. The target sequence is amplified according to the methods discussed above, wherein some of the nucleoside
5 triphosphates are biotin-labeled. This produces nucleotide sequences that are copies of the target sequence and contain one or more biotin-labeled nucleotides. The amplified sequences, which will be double-stranded, are then rendered single-stranded by known techniques,
10 preferably heat denaturation at a temperature of about 88°C to about 96°C. The sample is then contacted with immobilized probes. These probes are single-stranded polynucleotides attached to a solid support. Each one is capable of hybridizing with one of the single-stranded
15 nucleotide sequences. The probes are contacted with the sample for a sufficient time and under appropriate hybridizing conditions known to those skilled in the art to permit the polynucleotides to hybridize with the single-stranded nucleotide sequences. This forms bound complexes
20 of the polynucleotides and the single-stranded nucleotide sequences.

The probes can be prepared by standard techniques known to those skilled in the art, given the particular target sequence and the teachings contained herein. When
25 the nucleic acid is HIV-1 DNA, the preferred probe comprises the sequence 5' TTAAACACC ATGCTAAACA CAGT 3' (SEQ ID NO: 3), which we call the HOPE 3 probe. The HOPE 3 probe may also be used with a pyrimidine spacer at the 5' end. Preferably, the spacer is CTCTC, in which case we
30 call the probe HOPE 4.

- 20 -

The solid support may be any solid material to which the probe may be attached. Such material includes filters, resins, beads, cubes, and microtiter plates. Preferably, the solid supports are the wells in plastic
5 microtiter plates, such as polystyrene microtiter plates. Most preferably, the probe is first chemically coupled to a protein carrier, such as bovine serum albumin, which is then immobilized onto polystyrene microtiter plate wells. See Running and Urdea, BioTechniques, 8:276-277 (1990) and
10 Nagata, et al., FEBS Letters, 183:379-382 (1985), both of which are incorporated herein by reference.

The single-stranded polynucleotide of the probe is attached to the solid support covalently or noncovalently by means known to those skilled in the art. Preferably,
15 the probe is chemically coupled to a protein, such as bovine serum albumin, or other carrier, such as polyethylene glycol. A spacer between the carrier and the probe may also be used. The polynucleotide comprising the probe may contain from about 15 to about 2,000 nucleotides.
20 Preferably, it contains from about 15 to about 200 nucleotides and most preferably from about 15 to about 40 nucleotides.

The final step in this method of the invention is to detect or measure the presence of biotin in the bound
25 complex. One way of doing so is to contact the bound complexes with a detectable moiety that binds to the biotin and then to detect such moiety. Preferably, such detectable moiety is comprised of a molecule or compound that binds to the biotin, such as avidin or streptavidin,
30 coupled to a detectable entity (e.g., a detectable molecule or compound), such as horseradish peroxidase. The

- 21 -

horseradish peroxidase is detected by known means. Other detectable moieties/detectable entities are disclosed in U.S. patent No. 4,711,955 issued December 8, 1987 to Ward et al., which is incorporated herein by reference.

5 Preferably, the bound complexes are washed with a liquid, such as a buffer solution, before and/or after contact with the detectable moiety to remove any unbound, labeled sequences or labeled nucleoside triphosphates that may be present.

10 In a particular preferred embodiment, the invention provides a method for a two-stage amplification of the signal provided by the biotin. The method may be used to detect target nucleic acid sequences that have been amplified through the formation of primer extension
15 products that contain one or more biotin-labeled nucleotides. Thus, it is particularly applicable to the amplification methods of the present invention, but it is not limited to them.

 Such amplified sequences are rendered single-
20 stranded, and the sample containing such single-stranded sequences is contacted with immobilized probes as discussed above. The bound complexes comprising the single-stranded sequences and the single-stranded polynucleotides of the probes are then contacted with a first moiety that binds to
25 biotin. The moiety is preferably avidin or streptavidin. A second moiety, comprising biotin bound to a detectable moiety, is then added to the sample. The biotin in the second moiety binds to the first moiety, which is bound to the biotin in the amplified sequences. The detectable
30 moiety is then detected or measured by standard techniques.

- 22 -

Preferably, such detectable moiety is horseradish peroxidase. Preferably, the solid support is washed between the steps discussed above in order to remove unbound materials that may interfere with the detection
5 procedure or give a false signal.

The present invention also comprises a kit for detecting or measuring the presence of a target nucleic acid sequence in a sample suspected of containing that sequence. The kit comprises biotin-labeled nucleoside
10 triphosphates and an appropriate primer pair. Each primer comprises an oligonucleotide having a region that is complementary to and hybridizes with a different strand of the target sequence and is effective as a primer for nucleoside polymerization. Preferably, the kit further
15 comprises a probe that is complementary to and hybridizes with the target sequence. Most preferably, the kit further comprises a nucleic acid polymerase, preferably Taq polymerase, and additional means, such as reagents, for detecting or measuring the biotin, denaturing double-
20 stranded polynucleotides, and providing a control against which the results may be evaluated.

In a particularly preferred embodiment, the invention provides a kit for detecting or measuring the presence of a target nucleic acid sequence in HIV-1 DNA in
25 a sample. The kit contains biotin-labeled nucleoside triphosphates, unlabeled nucleoside triphosphates, a primer pair comprising the nucleotide sequences:

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCTGTA GTTCGTCGG 5' (SEQ ID NO: 2)

- 23 -

and a probe comprising the nucleotide sequence

5' TTAAACACC ATGCTAAACA CAGT 3'. (SEQ ID NO: 3)

5 It is to be understood that the application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products and processes of the present invention appear in the following examples.

EXAMPLE 1

10

Isolation of DNA From Blood

For AIDS screening, the object is a rapid and safe extraction of DNA to allow the processing of many samples. The suggested method is the following. Four hundred microliters (400 ul) of blood is collected in a tube
15 containing anti-clotting agent (citrate or EDTA) and is centrifuged (e.g. 3,000 g for 5 minutes or 10,000 g for 5-10 seconds). The upper buffy-coat layer is removed, or the whole pellet is suspended in 200 ul distilled water in order to lyse the cells. The lysed cell suspension is
20 boiled at 100°C in order to break up the cell nuclei. Centrifugation for 5 minutes at 10,000 g removes insoluble debris. The resultant supernatant is used for subsequent manipulations.

- 24 -

EXAMPLE 2One Temperature Reaction (CER)

Fifteen ul of DNA sample prepared as in Example 1 are added to 80 ul of a reaction buffer. The reaction
5 buffer contains 0.3nmol of each primer (HOPE 1, HOPE 2),
10mM Tris-HCl, pH 8.4 (20°C), 50mM KCl, 2mM MgCl₂, 0.1%
gelatin, 0.5mM dATP, dCTP, dGTP, 150 uM dTTP, 350 uM Bio-
11-dUTP, and 0.1% BSA (nuclease free). The reaction mix is
10 incubated at 95°C for five minutes. This is to initially
denature the DNA. The mix is then allowed to cool down to
room temperature, three units of the enzyme Vent polymerase
(New England Biolabs) are added, and mineral oil is layered
at the top. The reaction then takes place at 78°C for 3-4
hours.

15

EXAMPLE 3Two Temperature Reaction (ACER)

Ten microliters of the DNA containing supernatant prepared as in Example 1 are used for this amplification
reaction. This volume is mixed with 90 ul of reaction
20 buffer. The reaction buffer comprises 100pmol each primer
(melting temperature higher than 62°C, i.e., NIDA1, NIDA2),
10mM Tris-HCl, pH 8.0 (20°C), 1.8mM MgCl₂, 50 mM KCl, 0.05%
gelatin, 0.1mM dATP, dCTP, dGTP, 0.3nM dTTP, 70nM Bio-11-
dUTP, and 0.1% BSA (nuclease free).

25

The reaction mixture is then layered with one or
two drops of any mineral oil and transferred to a
programmable heat block where amplification takes place.

- 25 -

The temperatures used are 90°C for the initial and subsequent denaturation steps, and 70°C for annealing and elongation. The incubation period allowed for denaturation is one minute and for annealing, which is coupled with
5 elongation, two minutes. This thermal profile is repeated 20 times, with the annealing-elongation step of the 20th cycle being the last step of the reaction. Reaction products are stored at 4°C for further manipulation. The amplification reaction takes place in the wells of a 96-
10 well ELISA plate, a very convenient way when dealing with a large number of samples.

EXAMPLE 4

Two Temperature Asymmetric Reaction (AACER)

This type of reaction is essentially the same as
15 the ACER, except for the amount of the primers, which now is 20pmol for HOPE 1 and 300pmol for HOPE 2. The volume of the DNA sample is also increased to 20 ul.

EXAMPLE 5

Detection of Amplified Products

20 The totality (100 ul) of the amplification reaction volume (ACER or CER) is applied to a well containing immobilized probe after heat denaturation (100°C, five minutes) and snap cooling on ice. (Denaturation is not necessary when asymmetric two temperatures, or one
25 temperature reactions are performed.) Hybridization is allowed to proceed for one hour at 37°C. Unbound material is removed by washing four times with 0.0M NaCl.

- 26 -

A 50ng/ml solution of avidin-HRP in 10mM Tris-HCl pH 7.4 (20°C), 5% gelatin, and 2% BSA is added to the well. After incubating 30 minutes at room temperature with shaking, the well is washed with 0.9M NaCl, 10mM Tris-HCl, 5 pH 7.4 (20°C), and 0.1% Tween-20. The substrate mix is then applied (0.05% TetraMethylBenzidine, 10% H₂O₂). Blue color develops within 10 minutes. The colorigenic reaction is stopped with 0.5% H₂SO₄, and absorbance at 450nm is measured.

10

EXAMPLE 6Preparation of the Primers

The synthesis of the primers is standard and automated (Applied Biosystems synthesizer, model 380B). Synthesis is based on a solid phase chemistry where the 15 first base is already bound to a controlled pore glass (CPG) support at the 3' end. The 5' end is protected with a dimethoxytrityl group. Each new base is covalently coupled to the previous base at the 5' end using a complete synthesis cycle. The cycle consists of 4 steps:

20 1. Detritylation. The attached (terminal) base is detritylated with trichloroacetic acid creating a free hydroxyl group.

 2. Activation and addition. The next base is activated with tetrazole at the 3' end and then is passed 25 through the CPG column allowing it to react with 5' hydroxyl of the terminal base.

- 27 -

3. Capping. The unreacted 5' hydroxyl groups (approximately 0-2%) are blocked with acetic anhydride (acetylation).

5 4. Oxidation. The phosphorous of the new base is oxidized from the trivalent to the pentavalent form using a iodine-water-lutidine-tetrahydrofuran mixture.

After the completion of the synthesis cycle, the new base is accessible for the repeat of the cycle with the next base. Thus, elongation of the DNA chain is achieved
10 with the correct nucleotide sequence.

At the end of the run, the product is cleaved from the CPG using 25% ammonia solution. The bases of the resultant oligonucleotide are then deprotected by heat-treatment at 55°C in the presence of ammonia for 5 hours.

15 The product is evaporated to dryness to remove the ammonia, reconstituted in water and dried again, and finally reconstituted in water. The product is stored at -20°C until further use.

EXAMPLE 7

20

Preparation of the Probe

The oligonucleotide HOPE4 (5' CTCTCTTTAA ACACCATGCT AACACAGT 3') (SEQ ID NO:6) was synthesized using a DNA synthesizer (Applied Biosystems). It was then chemically coupled to bovine serum albumin, which was immobilized into
25 the wells of a polystyrene microtiter plate according to

- 28 -

the method of Running and Urdea, op. cit., and Nagata, et al., op. cit.

EXAMPLE 8

Evaluation of Samples

5 Eight seropositive samples were examined, plus a DNA sample prepared from an infected culture of CEM cells. Several seronegative samples were also examined. A positive control-plasmid derived cloned sequences of HIV-I, and a negative control, were always included.

10 Some samples were analyzed by applying CER and others by ACER. Amplification products were detected by either in solution hybridization with a specific radiolabeled probe (HOPE 3) and subsequent acrylamide gel electrophoresis or the ELISA-like colorimetric assay using
15 as label a biotinylated dNTP analog or a labeled primer (HOPE 2).

The values obtained were as follows:

Absorbance readings of ELISA-like assay:

	1 POS)	0.40	seropositive (5 yrs)
20	2 POS)	0.30	seropositive (1 yr)
	3 POS)	0.60	dead
	4 POS)	0.20	seropositive (1.5 yrs)
	5 POS)	0.40	under AZT (4 yrs)
	6 POS)	0.55	dead
25	7 POS)	0.30	seropositive (2 yrs)
	8 POS)	0.20	seropositive (1 yr)

- 29 -

	9 CEM)	0.60	infected cell line
	10 CONT)	1.00	plasmid clone of HIV-I
	11 CONT)	0.06	yeast DNA
	12 CONT)	0.05	no DNA
5	13 NEG)	0.07	seronegative
	14 NEG)	0.06	seronegative

- 30 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANTS: Nectarios Tavernarakis,
George Hatzidakis, and
Elias Krambovitis
- (ii) TITLE OF INVENTION: RAPID AMPLIFICATION
AND DETECTION OF
NUCLEIC ACIDS
- (iii) NUMBER OF SEQUENCES: 5
- 10 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Dickstein, Shapiro &
Morin
(B) STREET: 2101 L Street, N.W.
(C) CITY: Washington
(D) STATE: D.C.
15 (E) COUNTRY: U.S.A.
(F) ZIP: 20037
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII

- 31 -

- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 5 (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Karny, Geoffrey M.
10 (B) REGISTRATION NUMBER: 31,382
(C) DOCKET NUMBER: I2277.001/P001
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 785-9700
(B) TELEFAX: (202) 887-0689
- 15 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 nucleotides
(B) TYPE: nucleic acid
20 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGGAGCCA CCCCACAAG

19

- 32 -

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION; SEQ ID NO:2:

CCCCCCTGTA GTTCGTCGG 19

10 (4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTAAACACC ATGCTAAACA CAGT 24

(5) INFORMATION FOR SEQ ID NO:4:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
(B) TYPE: nucleic acid

- 33 -

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5

GACATCGAGC TTGCTAGAAG

20

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

10

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGACGAAT TCGGAGTTAT

20

15 (7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 nucleotides

(B) TYPE: nucleic acid

20

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

- 34 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTCTTTAA ACACCATGCT AACACAGT 28

WE CLAIM:

1. A method for amplifying a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

5 (a) adding nucleoside triphosphates, primer pairs comprising two oligonucleotide primers, and a nucleic acid polymerase to said sample;

(b) denaturing said target nucleic acid sequence to form separate strands; and

10 (c) maintaining a reaction temperature in a range from about 68°C to about 80°C and appropriate reaction conditions wherein the following cycle occurs: said primers hybridize to said strands of said target sequence, primer extension products, which are hybridized to said strands, are formed from said primers and said
15 nucleoside triphosphates, said extension products separate from said strands to become templates for said primers, and new primer extension products are formed.

2. The method of claim 1 wherein said target sequence is denatured by heating said sample to about 95°C.

20 3. The method of claim 1 wherein the reaction temperature in step (c) is maintained at about 75°C.

4. The method of claim 1 wherein said primers bind strongly to the strands of said target sequence and the extensions from said primers bind weakly to the strands
25 of said target sequence.

5. The method of claim 4 wherein the ratio of G and C to A and T in said primers ranges from about 1.5:1 to about 3.0:1.

6. The method of claim 5 wherein said ratio is
5 about 3.0:1.

7. The method of claim 5 wherein the length of said primers is about 15-25 nucleotides.

8. The method of claim 5 wherein the concentration of one of said primers in said primer pair
10 exceeds the concentration of the other primer by a factor of about 10^3 to 1 to about 10^2 to 1.

9. The method of claim 1 wherein the cycle of step (c) is repeated about 10 to about 25 times.

10. The method of claim 1 wherein the reaction
15 temperature and conditions of step (c) are maintained for a time from about 1.0 hours to about 2.5 hours.

11. The method of claim 1 wherein said nucleic acid is AIDS-related viral nucleic acid.

12. The method of claim 1 wherein said target
20 nucleic acid is RNA and said sample is treated with reverse transcriptase.

13. The method of claim 1 wherein said target nucleic acid is DNA and said nucleoside triphosphates are deoxynucleoside triphosphates.

- 37 -

14. The method of claim 13 wherein said DNA is HIV-1 DNA.

15. The method of claim 14 wherein said target sequence is the nucleotide sequence 1317-1379 of pHXB2.

5 16. The method of claim 15 wherein said primer pair comprises the nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5'. (SEQ ID NO: 2)

10 17. A method for detecting or measuring a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

15 (a) amplifying said target sequence according to claim 1, wherein some of said nucleoside triphosphates are biotin-labeled, thereby producing nucleotide sequences that are copies of said target nucleotide sequence and contain one or more biotin-labeled nucleotides;

(b) rendering said nucleotide sequences single-stranded;

20 (c) contacting said sample containing said single-stranded nucleotide sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide

- 38 -

sequences, for a sufficient time and under appropriate hybridizing conditions to permit said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and
5 said single-stranded nucleotide sequences; and

(d) detecting or measuring the presence of biotin in said bound complexes.

18. The method of claim 17 wherein said solid support is a microtiter plate.

10 19. The method of claim 17 wherein said detecting or measuring step comprises the steps of:

contacting said bound complexes with a detectable moiety that binds to biotin; and

detecting or measuring said detectable moiety.

15 20. The method of claim 19 wherein said detectable moiety is avidin-horseradish peroxidase.

21. The method of claim 17 wherein said detecting or measuring step comprises the steps of:

20 contacting said bound complexes with a first moiety that binds to biotin;

adding a second moiety comprising biotin bound to a detectable moiety, whereby the biotin in said second moiety binds to said first moiety; and

detecting or measuring said detectable moiety.

22. The method of claim 21 wherein said first moiety is avidin and said detectable moiety is horseradish peroxidase.

5 23. The method of claim 19 wherein said nucleic acid is HIV-1 DNA and said single-stranded polynucleotide of said probe comprises the sequence

5' TTAAACACC ATGCTAAACA CAGT 3' (SEQ ID NO: 3) or

5' CTCTCTTTAA ACACCATGCT AACACAGT 3' (SEQ ID NO: 6).

10 24. A method for amplifying a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

(a) adding nucleoside triphosphates, primer pairs comprising two oligonucleotide primers, and a nucleic
15 acid polymerase to said sample;

(b) denaturing said target nucleic acid sequence to form separate strands;

(c) maintaining the reaction temperature in a range from about 68°C to about 82°C and appropriate
20 reaction conditions to form primer extension products from said primers and said nucleoside triphosphates, wherein said extension products are hybridized to said strands;

- 40 -

(d) separating said extension products from said strands by raising the temperature to a range of about 88°C to about 96°C to produce single-stranded molecules;

5 (e) lowering the reaction temperature to a range from about 68°C to about 82°C and maintaining appropriate reaction conditions to synthesize primer extension products from said primers and said nucleoside triphosphates using the single-stranded molecules produced by step (d) as a template; and

10 (f) repeating steps (d) and (e) a sufficient number of times to obtain the desired amplification of said target nucleic acid sequence.

25. The method of claim 24 wherein the temperature at which step (d) is performed is about 90°C and the
15 temperature at which steps (c) and (e) is performed is about 70°C.

26. The method of claim 24 wherein said primers bind strongly to the strands of said target sequence and the extensions from said primers bind weakly to the strands
20 of said target sequence.

27. The method of claim 26 wherein the ratio of G and C to A and T in said primers ranges from about 1.5:1 to about 3.0:1.

28. The method of claim 24 wherein the reaction
25 time for steps (c)-(f) is from about 0.5 hours to about 3 hours.

- 41 -

29. The method of claim 24 wherein said nucleic acid is HIV-1 DNA and said nucleoside triphosphates are deoxynucleoside triphosphates.

30. The method of claim 29 wherein said target
5 sequence is the nucleotide sequence 1317-1379 of PHXB2.

31. The method of claim 30 wherein said primer pair comprises the nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5'. (SEQ ID NO: 2)

10 32. The method of claim 29 wherein said target sequence is the nucleotide sequence 331-530 of PHXB2.

33. The method of claim 32 wherein said primer pair comprises the nucleotide sequences

5' GACATCGAGC TTGCTAGAAG 3' (SEQ ID NO: 4)

15 3' GGTGACGAAT TCGGAGTTAT 5'. (SEQ ID NO: 5)

34. A method for detecting or measuring a target nucleic acid sequence in a sample, wherein said target sequence consists of two complementary strands, comprising the steps of:

20 (a) amplifying said target sequence according to claim 25, wherein some of said nucleoside triphosphates are biotin-labeled, thereby producing nucleotide sequences

- 42 -

that are copies of said target nucleotide sequence and contain one or more biotin-labeled nucleotides;

(b) rendering said nucleotide sequences single-stranded;

5 (c) contacting said sample containing said single-stranded nucleotide sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide
10 sequences, for a sufficient time and under appropriate hybridizing conditions to permit said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and said single-stranded nucleotide sequences; and

15 (d) detecting or measuring the presence of biotin in said bound complexes.

35. The method of claim 34 wherein said detecting or measuring step comprises the steps of:

contacting said bound complexes with a
20 detectable moiety that binds to biotin; and

detecting or measuring said detectable moiety.

36. The method of claim 34 wherein said nucleic acid is HIV-1 DNA and said single-stranded polynucleotide of said probe comprises the sequence

25 5' TTAAACACC ATGCTAAACA CAGT 3'. (SEQ ID NO: 3)

- 43 -

37. A method for detecting or measuring the presence of a target nucleic acid sequence in a sample comprising the steps of:

- 5 (a) amplifying said target nucleic acid sequence through the formation of primer extension products that contain one or more biotin-labeled nucleotides;
- (b) rendering said primer extension products single-stranded;
- 10 (c) contacting said sample containing said single-stranded sequences with immobilized probes, each of which comprises a single-stranded polynucleotide attached to a solid support and capable of hybridizing with one of said single-stranded nucleotide sequences, for a sufficient time and under appropriate hybridizing conditions to permit
15 said polynucleotides to hybridize with said single-stranded nucleotide sequences, thereby forming bound complexes of said polynucleotides and said single-stranded nucleotide sequences;
- 20 (d) contacting said bound complexes with a first moiety that binds to biotin;
- (e) adding a second moiety, comprising biotin bound to a detectable moiety, wherein the biotin in said second moiety binds to said first moiety; and
- 25 (f) detecting or measuring said detectable moiety.

38. The method of claim 37 wherein said first moiety is avidin and said detectable moiety is horseradish peroxidase.

39. The method of claim 37 wherein said amplifying
5 step is performed according to claim 1.

40. The method of claim 37 wherein said amplifying step is performed according to claim 24.

41. A primer pair for use in amplifying a target nucleic acid sequence of HIV-1 DNA comprising the
10 nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5'. (SEQ ID NO: 2)

42. A primer pair for use in amplifying a target nucleic acid sequence of HIV-1 DNA comprising the
15 nucleotide sequences

5' GACATCGAGC TTGCTAGAAG 3' (SEQ ID NO: 4)

3' GGTGACGAAT TCGGAGTTAT 5'. (SEQ ID NO: 5)

43. A probe for use in detecting a nucleotide sequence complementary to a target nucleic acid sequence of
20 HIV-1 DNA, said probe comprising the nucleotide sequence

5' TTAAACACC ATGCTAAACA CAGT 3' (SEQ ID NO: 3) or

5' CTCTCTTTAA ACACCATGCT AACACAGT 3' (SEQ ID NO: 6).

44. A kit for detecting or measuring the presence of a target nucleic acid sequence in a sample suspected of containing said sequence comprising, in a container:

5 (a) biotin-labeled nucleoside triphosphates;
and

(b) a primer pair consisting of two oligonucleotide primers, each primer comprising an oligonucleotide having a region that is complementary to
10 and hybridizes with a different strand of said target sequence and being effective as a primer for nucleoside polymerization.

45. The kit of claim 44 further comprising a probe
15 that is complementary to and hybridizes with said target sequence.

46. The kit of claim 45 further comprising means for detecting or measuring said biotin.

47. The kit of claim 46 further comprising a nucleic acid polymerase.

20 48. The kit of claim 44 wherein said primer pair comprises the nucleotide sequences

5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCCTGTA GTTCGTCGG 5'. (SEQ ID NO: 2)

49. The kit of claim 44 wherein said primer pair comprises the nucleotide sequences

5' GACATCGAGC TTGCTAGAAG 3' (SEQ ID NO: 4)

3' GGTGACGAAT TCGGAGTTAT 5'. (SEQ ID NO: 5)

5 50. The kit of claim 45 wherein said probe comprises the nucleotide sequence

5' TTAAACACC ATGCTAAACA CAGT 3'. (SEQ ID NO: 3)

10 51. A kit for detecting or measuring the presence of a target nucleic acid sequence in HIV-1 DNA in a sample suspected of containing said sequence comprising, in a container:

(a) biotin-labeled nucleoside triphosphates;

(b) a primer pair comprising the nucleotide sequences

15 5' GAAGGAGCCA CCCCACAAG 3' (SEQ ID NO: 1)

3' CCCCCTGTA GTTCGTCGG 5' (SEQ ID NO: 2)

and

(c) a probe comprising the nucleotide sequence

20 5' TTAAACACC ATGCTAAACA CAGT 3'. (SEQ ID NO: 3)

INTERNATIONAL SEARCH REPORT

International application No.

F /GR 92/00015

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12Q 1/68, C12Q 1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF INFECTIOUS DISEASES, Volume 161, No, 1990, Kathleen D. Eisenach et al, "Polymerase Chain Reaction Amplification of a Repetitive DNA Sequence Specific for Mycobacterium tuberculosis", see especially p 978, col. 1, lines 1-10 --	1-14, 17-22, 24-30, 34-35, 37-40, 44-47
X	J CLIN PATHOL, Volume 45, No, 1992, E S-F Lo et al, "Detection of hepatitis B pre-core mutant by allele specific polymerase chain reaction", see especially p. 690 --	1-14, 17-22, 24-30, 34-35, 37-40, 44-47

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family


Date of the actual completion of the international search

Date of mailing of the international search report

10 February 1993

25.02.93

Name and mailing address of the ISA/


 European Patent Office, P.B. 5818 Patentlaan 2
 NL-2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Mikael G:son Bergstrand

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GR 92/00015

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	EP, A1, 0511712 (EASTMAN KODAK COMPANY), 4 November 1992 (04.11.92) -- -----	1-47

SA 2772

INTERNATIONAL SEARCH REPORT

Information on patent family members

08/01/93

International application No.

PCT/GR 92/00015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0511712	04/11/92	NONE	